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Vitamin A supplementation enhances Senegalese sole (*Solea senegalensis*) early juvenile's immunocompetence: new insights on potential underlying pathways

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Abstract :

Senegalese sole (*Solea senegalensis*) has been considered since the 1990's to be a promising flatfish species for diversifying European marine aquaculture. However, pathogen outbreaks leading to high mortality rates can impair Senegalese sole commercial production at the weaning phase. Different approaches have been shown to improve fish immunocompetence; with this in mind the objective of the work described herein was to determine whether increased levels of dietary vitamin A (VA) improve the immune response in early juveniles of Senegalese sole. For this purpose, Senegalese sole were reared and fed with *Artemia* metanauplii containing increased levels of VA (37,000; 44,666; 82,666 and 203,000 total VA IU Kg⁻¹) from 6 to 60 days post-hatch (early juvenile stage). After an induced bacterial infection with a 50 % lethal dose of *Photobacterium damsela* subsp. *damsela*, survival rate, as well as underlying gene expression of specific immune markers (*C1inh*, *C3*, *C9*, *Lgals1*, *Hamp*, *LysC*, *Prdx1*, *Steap4* and *Transf*) were evaluated. Results showed that fish fed higher doses of dietary VA were more resistant to the bacterial challenge. The lower mortality was found to be related with differential expression of genes involved in the complement system and iron availability. We suggest that feeding metamorphosed Senegalese sole with 203,000 total VA IU Kg⁻¹ might be an effective, inexpensive and environmentally friendly method to improve Senegalese sole immunocompetence, thereby improving survival of juveniles and reducing economic losses.

Highlights

► Dietary vitamin A (VA) enhances immune system in *Solea senegalensis* juveniles. ► Sole fed 203,000 VA IU Kg⁻¹ had higher resistance to an induced bacterial infection. ► Complement system and iron homeostasis genes were differentially expressed. ► Dietary VA levels impact on the fish osteoimmunological response.

Keywords : vitamin, retinoic acid, immune system, gene expression, Senegalese sole *Solea senegalensis*, *Photobacterium damsela* subsp. *damsela*

49 1. Introduction

50 Diversification of marine aquaculture is a major issue, with Senegalese sole (*Solea*
51 *senegalensis*) being one of the most promising species for aquaculture diversification in the
52 Iberian Peninsula [1]. As recently reviewed [1], an increased understanding has been obtained
53 on the manner in which husbandry practices, environmental conditions (such as rearing
54 temperature, salinity, light spectrum and intensity), genetic background and/or nutrition
55 influences fish physiology. However, despite this research effort, multiple bottlenecks like
56 resistance to pathogen infection, outbreaks of infectious disease, incidence of skeletal
57 deformities, and impaired reproductive performance in captivity still exist [1].

58 In Senegalese sole farms, a wide array of pathogens has been detected [2], which are
59 commonly treated with antibiotics and/or chemotherapeutics. In particular, *Photobacterium*
60 *damselfae* subsp. *piscicida* (*Phdp*), the aetiologic agent of fish pasteurellosis, induces economic
61 losses [3] and thus, has received significant attention. In contrast, less is known regarding *P.*
62 *damselfae* subsp. *damselfae* (*Phdd*), although it is an emerging pathogen in marine aquaculture
63 [4].

64 Nowadays, more environmentally friendly aquaculture strategies to fight bacterial infections
65 are being sought. In this context, probiotics as well as other immunomodulatory substances like
66 vitamins, minerals and amino acids seem to offer an attractive alternative [5, 6] to
67 chemotherapies. Vitamin A (VA), and its derivatives (retinoids), have pleiotropic roles. Since
68 fish are not able to synthesize VA compounds *de novo*, they have to acquire them from the
69 diet. Their excess or deficiency cause abnormal development/homeostasis in different tissues,
70 organs and/or systems [7]. In particular, VA is widely known to promote the mammalian
71 immune response [8], although the mechanism by which VA effects this is still not clearly
72 understood. In different fish species, several VA dose-response studies have been conducted
73 to establish the minimum and optimal requirements for the development of some specific
74 tissues/organs [9-17], and suggesting that those requirements seem to be specific for different
75 cell types, tissues, developmental stages and fish species [12]. In contrast, limited work has

76 been carried out for deciphering the roles and requirements of different VA metabolites in fish
77 immunocompetence [18-23]. Thus, the present study aimed at investigating the effects of
78 different levels of dietary VA content on Senegalese sole immunocompetence against an
79 induced bacterial infection. Furthermore, an expression analysis of representative immune-
80 related genes by relative quantitative PCR (qPCR) has been performed in order to gain some
81 insights on how dietary VA content could affect Senegalese sole resistance to bacterial
82 infection.

83

84 **2. Materials and methods**

85 *2.1 Ethics statement*

86 Present work was carried out in accordance with EU Directive 2010/63/EU for animal
87 experiments (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm),
88 following approved protocols by the institutional ethics committee.

89

90 *2.2 Fish rearing and experimental diets*

91 Newly hatched larvae of Senegalese sole were obtained from Stolt Sea Farm SA (Cambre, La
92 Coruña, Spain) and shipped to IRTA facilities. After their acclimatization, larvae were
93 distributed in cylindrical tanks (100 L) connected to a recirculation unit (IRTAMar™).
94 Senegalese sole larvae were reared and fed enriched rotifers (Easy Selco™) first from 3 to 10
95 days post hatch (dph), after which from 6 dph onwards, larvae were fed with *Artemia*
96 metanauplii containing increased levels of VA (37,000, 44,666, 82,666 and 203,000 total VA IU
97 kg⁻¹; labeled "Control", VA1, VA2 and VA3, respectively) by adding different amounts of retinyl
98 palmitate (1,600,000 IU g⁻¹, Sigma-Aldrich, Spain) to a commercial enrichment emulsion (Easy
99 Selco™), as previously described [15]. From 33 dph onwards early juveniles were fed with
100 frozen *Artemia* metanauplii enriched according to the procedures mentioned above.

101

102 *2.3 Bacterial challenges*

103 Inoculum of *Photobacterium damsela* subsp. *damsela* (ATCC® 33539) (*Phdd*) was grown at
104 22 °C in tryptic soy broth (TSB) or tryptic soy agar (TSA) supplemented with NaCl to a final
105 concentration of 2% (w/v), then stored at -80 °C in TSB supplemented with 15% (v/v) glycerol.
106 To prepare the inocula for intramuscular injection, frozen stock of bacteria were inoculated into
107 10 mL of TSB medium with 2% NaCl and cultured for 24 h at 22 °C with continuous shaking
108 (90 rpm). Then, bacteria were inoculated onto TSA medium with 2% NaCl overnight at the
109 same temperature. Colonies from exponentially growing bacteria were resuspended in sterile
110 sea water (SSW) with 2.5% NaCl and the density of the culture was adjusted to McFarland
111 standard of 0.5. From this suspension, 10-fold dilutions ranging from 2×10^1 to 2×10^6 CFU mL⁻¹
112 were prepared. Aliquots of the serial dilutions were plated onto TSA 2% NaCl and the number
113 of CFU were counted following incubation at 22 °C in order to confirm CFU concentrations. A
114 standard curve for this bacterium was developed by plotting optical density at 610 nm against
115 bacterial counts (CFU mL⁻¹).

116 Three different challenge trials were performed in this study. In the first trial, the
117 determination of the LD₅₀ for *Phdd* in Senegalese sole early juveniles (LD₅₀ – *challenge*) was
118 conducted in 45 dph fish fed with 37,000 total VA IU kg⁻¹ (Control group). Juveniles were
119 inoculated intramuscularly (10 fish per 1 L aquarium) with 10 µL containing 0 (C), 6×10^1 , 6×10^2 ,
120 6×10^3 , 6×10^4 and 6×10^5 CFU mL⁻¹ suspended in SSW. Each dose was done in quadruplicate. A
121 second bacterial challenge trial was conducted to determine the resistance to an induced
122 bacterial infection of juveniles (60 dph; 15.4 ± 1.2 mm in standard length) when fed with
123 increasing dietary VA levels (*VA – challenge*). Thus, fish from each dietary VA group ($n = 7$)
124 were intramuscularly injected (10 µL) with the LD₅₀ previously calculated (6×10^2 CFU mL⁻¹) in
125 quadruplicate. In addition, a Control group was also injected with 10 µL of SSW (Control -
126 Control group). A third bacterial challenge trial was also implemented to get some insights on
127 how VA supplementation could modulate the immunocompetence of Senegalese sole juveniles
128 (75 dph). For this purpose 30 fish from the Control and VA3 groups were inoculated as
129 described in the *VA - challenge* and 5 fish were sampled at 4, 8, 12 and 24 h post-injection

130 (*Underlying pathways – challenge*). In all trials, fish were fed daily with frozen *Artemia*
131 previously enriched with their respective VA level, while water temperature was maintained at
132 18.0 ± 1.0 °C and with a 50 % water renewal in aquaria. Mortality was recorded on a daily
133 basis up to 18 days post injection. Survival rate (%) was calculated as the final number of
134 inoculated fish / initial number of inoculated fish * 100. All sampled fish were euthanized with
135 an overdose of anaesthetic (Tricaine methanesulfonate, MS-222, Sigma). Fish sampled during
136 the *Underlying pathways – challenge* were individually frozen in RNA later (Ambion®) and
137 stored at -80 °C until gene expression analysis. Additionally, to confirm *Phdd*
138 presence/absence, several moribund *Phdd* infected fish, as well as healthy SSW injected
139 specimens were sampled, washed in SSW, directly frozen and stored at -20 °C until DNA
140 extraction.

141

142 2.3 DNA extraction and confirmation of presence of *P. damselae* subsp. *damselae* by PCR

143 Total DNA isolation from fish specimens (infected or not) was performed following a proteinase
144 K lysis buffer protocol. *Phdd* presence/absence were then confirmed by PCR (initial
145 denaturation at 95 °C for 8 min, followed by 40 cycles of a 1 min denaturation at 94 °C,
146 annealing at 58 °C for 1 min, elongation at 72 °C for 1 min and a final extension of 10 min at 72
147 °C) with 16S-like ssrDNA degenerate primers (Supplementary Table S1) and subsequent
148 sequencing of the resulting amplicon.

149

150 2.4 RNA extraction, reverse transcription and gene expression analysis

151 From 3 injected fish collected at each sampling time and from both dietary groups (Control and
152 VA3) total RNA was extracted using TRIzol reagent (Invitrogen®) following manufacturer
153 specifications. The quantity of RNA isolated was determined using a Gene-Quant
154 spectrophotometer (Amersham Biosciences), purity was established by the absorbance ratio
155 260/280 nm (ranging from 1.7 to 2.0 in all samples). The quality of the RNA was examined
156 using 1.2% TAE agarose gel electrophoresis. A reverse transcription reaction was carried out

157 using equal quantities of total RNA (1 µg) from each sample and Quanti Tect Reverse
158 Transcription Kit (Qiagen®). Electrophoresis using a 1.2% TAE agarose gel was run to assess
159 the RT-PCR product. Real-time qPCR was performed using an ABI PRISM 7300 (Applied
160 Biosystems). For each gene, species and gene-specific primers were designed according to
161 sequences available in GenBank (NCBI, www.ncbi.nlm.nih.gov) and the SoleaDB databases
162 (www.aquagenet.eu; Supplementary Table S1). The efficiencies of all qPCR reactions were
163 close to 100%. qPCR reactions were performed in triplicate in 96-well plates containing: 10 µL
164 of SYBR® Green PCR Master Mix (Life Technologies), 0.5 µL of forward and reverse primers
165 (10 µM), 7 µL of molecular biology grade water (Sigma), and 2 µL of a 1:10 dilution of cDNA
166 template. Standard amplification parameters were: 95 °C for 5 min followed by 45 amplification
167 cycles (95 °C for 45 s, 59 °C for 1 min and 70 °C for 1min). A final dissociation reaction (melting
168 curve) was performed (95 °C for 15 s, 60 °C for 20 s, 95 °C for 15 s and 60 °C for 15 s) to
169 evaluate the specificity of the amplicons. A calibrator sample was included in each qPCR plate
170 [24].

171

172 *2.5 Data Processing*

173 Relative gene expression was determined from the ratio between PCR efficiency and threshold
174 cycle of a sample compared with the control, and expressed in comparison to the reference
175 gene, according to Pfaffl's mathematical model [25]. Relative gene expression was normalized
176 using ubiquitin (*Ubp*), a previously reported reference gene for accurate normalization in qPCR
177 studies with Senegalese sole [26], and using the gene expression level of the Control group as
178 reference sample.

179

180 *2.6 Statistical analysis*

181 Results are given as mean ± standard deviation. All data were checked for normality
182 (Kolmogorov–Smirnov test) and homoscedasticity of variance (Bartlett's test). Survival rate

183 expressed in percentage was arcsin square root transformed before statistical analyses.
184 Significant differences in survival rate and in gene expression ratios during the time course of
185 infection (0-24 h post injection) in fish fed with Control diet were detected by one-way ANOVA,
186 and by T-test at each sampling time between the Control and VA3 dietary groups. When
187 differences were significant by the ANOVA test, the Tukey multiple-comparison test was used
188 to detect differences among experimental groups. Differences were considered to be significant
189 when $P < 0.05$. The correlation between fish mortality and total VA levels in enriched *Artemia*
190 metanauplius was evaluated by means of the Pearson Product Moment Correlation test. All
191 statistical analyses were conducted using SigmaStat 3.0 (Systat Software Inc.).

192

193 **3. Results and discussion**

194 Although *Phdd* is a moderately virulent pathogen, it is one of the most common pathogens
195 associated with newly cultured fish species [4, 27]. In the present study, we found a LD₅₀ of 6
196 CFU per fish in Senegalese sole early juveniles (around 10-20 mg dry weight; Supplementary
197 Figure S1) and therefore, this bacterial load was applied subsequently to decipher whether
198 dietary VA level improved Senegalese sole early juvenile's immunocompetence.

199

200 *3.1 Dietary VA supplementation improve the resistance of Senegalese sole early juveniles to* 201 *an induced bacterial infection*

202 The intensive culture of fish species has favored the occurrence of several outbreaks with
203 *Photobacterium damsela* subsp. *damsela* resulting in moderate mortality [30]. Despite
204 increasing knowledge about *Phdp* pathogenesis, relatively little is yet known about how *Phdd*
205 induces fish mortality (reviewed in Labella et al. [4]). Further, the effectiveness of
206 chemotherapy treatments remains limited [28, 29] when confronted with isolates of *Phdp* and
207 *Phdd* possessing multidrug resistance genes which can be transferred horizontally. Therefore,
208 immunoprophylaxis seems to be the best way to prevent pasteurellosis. Autovaccines against
209 *Phdp* have been used in some farms [1], but their efficacy is dependent on the fish species,

210 fish size, vaccine formulation and immunostimulants used [30]. In addition, recent studies on
211 probiotics to control Photobacteriosis have given encouraging results [31, 32]. Under current
212 experimental conditions, Senegalese sole early juveniles fed with a VA-supplemented diet
213 (203,000 total VA IU Kg⁻¹) showed a higher resistance to an induced bacterial infection with
214 *Phdd* (Fig. 1; ANOVA, $P < 0.05$), with approximately a 50% reduction in endpoint mortality in
215 comparison to the Control group (37,000 total VA IU Kg⁻¹; 36.6 ± 11.8 and 89.3 ± 13.7 % of
216 cumulative mortality, respectively); and a statistically significant negative correlation between
217 endpoint mortality rates and total VA dietary content ($R = -0.971$; $P = 0.02$; Pearson Product
218 Moment Correlation test). This confirmed that VA has a role in Senegalese sole
219 immunocompetence. Similar results were found regarding the enhancement of the immune
220 system by retinoids in freshwater [18-20] and marine fish species [21-23]. In order to confirm
221 that fish mortality was caused by the intramuscular injection of *Phdd*, moribund challenged fish
222 and healthy control ones injected with SSW were sampled. Confirmation of presence/absence
223 of *Phdd* was done by PCR (Supplementary Figure S2).

224

225 3.2 VA might ameliorate the response to *Phdd* infection through different immune pathways

226 *Phdd* induced pathogenicity and mortality are known to be caused by excreted cellular protein
227 components such as proteases, hemolysins, and siderophore-mediated iron sequestering
228 proteins (reviewed in Labella et al. [4]). However, how the fish immune system responds to
229 this pathogen is still unknown. Thus, the time course response to *Phdd* infection in sole
230 juveniles fed the Control and the highest VA supplemented (VA3) diets was evaluated by
231 analyzing the expression of genes from the immune system.

232 All evaluated genes showed no differences in their expression levels between fish fed the
233 Control and VA3 diets before the bacterial challenges (Figs. 2 and 3; T-test, $P > 0.05$),
234 indicating that VA might prime the system to give an enhanced response upon stimulation by
235 pathogen infection [33]. It is known that VA metabolites improve the immunocompetence in
236 vertebrates [33]. Fish fed with synthetic or natural β -carotene and astaxanthin supplemented

237 diets had a higher serum complement activity, lysozyme activity, phagocytic activity and better
238 non-specific cytotoxicity in peripheral blood lymphocytes, increasing the bio-defense
239 mechanisms of the organism [18-20]. Furthermore, fish fed diets supplemented with
240 astaxanthin showed higher resistance against infectious hematopoietic necrosis virus (IHNV)
241 [20], while fish fed with high levels of retinol acetate, respiratory burst activity, leukocyte
242 cytotoxic activity and total serum immunoglobulin M were enhanced [21-23].

243 Although additional analyses are required, the present work showed some potential
244 underlying pathways by which VA might ameliorate fish immunocompetence. At 24 h post
245 infection (hpi) a higher gene expression (ANOVA, $P < 0.05$) of the three main molecular players
246 of the complement system was observed (Figure 2): *complement component 1 inhibitor (C1inh*;
247 *from the classical and lectin pathway)*, *complement component 3 (C3*; *from the alternative*
248 *pathway)* and *complement component 9 (C9*; *from the cytolytic pathway)*. Those findings were
249 in partial agreement with those reported in Senegalese sole [34] and other fish species [35]
250 after an infection with *Phdp*, where it was proposed that activation of complement provided
251 protection against *Phdp* infection [35]. The common response among fish species (activation of
252 complement) against multiple pathogens suggests that the supplementation of diet with VA can
253 be used as an immunoprophylactic measure in different fish species and against more than
254 one pathogen.

255 A higher expression of *C1inh* was observed in fish fed with supplemented VA diets at 8 and
256 12 hpi compared with that of infected soles fed with the Control diet (Fig. 2; T-test, $P < 0.05$).
257 Interestingly, the classical and lectin pathway mainly relies on the activation of the C1 complex,
258 which is under control of the C1-inhibitor that forms a proteolytically inactive stoichiometric
259 complex with the C1r or C1s proteases. Although activation of the C1 complex plays crucial
260 roles for successful host defense, excessive activation of complement can potentially cause
261 serious damage to the host, leading to anaphylaxis and cell damage [36]. Similarly, a higher
262 gene expression of *C3* in Senegalese sole fed VA3 diet was also reported, although it was only
263 significant at 12 hpi (Fig. 2; T-test, $P < 0.05$). Complement *C3* is the central protein of all three

264 activation pathways and essential in alerting the host of the presence of potential pathogens,
265 contributing significantly to the orchestration and development of an acquired immune
266 response [36, 37]. The highest gene expression of C3 in Senegalese sole was found in the
267 group fed with Control diet at 24 hpi, which is in accordance with results reported in different
268 fish species after 24 h infection with *Phdp* [38]. In contrast, C9 up-regulation in infected fish
269 started at 4 hpi, but was only seen to be significantly different at 24 hpi in VA3 group. The ninth
270 component of the complement is a single-chain glycoprotein that is involved in the formation of
271 the membrane attack complex (MAC) on the surface of target cells [38].

272 The current study also provides new insights on other potential underlying pathways by
273 which dietary VA content can improve fish immunocompetence. In this sense, expression of
274 *lysozyme C (LysC)*, *lectin galactoside-binding soluble 1 (galectin 1)-like 3 (Lgals1)*,
275 *peroxiredoxin-1 (Prdx1)*, also known as *natural killer cell-enhancing factor*, *hepcidin (Hamp)*,
276 *transferrin (Transf)*, and *metalloreductase six-transmembrane epithelial antigen of prostate 4*
277 (*Steap4*; also known as *tumor necrosis factor, alpha-induced protein 9*) were also evaluated.
278 Lysozymes exhibit antimicrobial properties by hydrolysis of N-acetylmuramic acid and N-
279 acetylglucosamine, which are constituents of the peptidoglycan layer of bacterial cell walls [39].
280 Our results showed that from the initiation of infection onwards, *LysC* was significantly down-
281 regulated in both experimental groups (Control and VA3; Fig. 3; ANOVA, $P < 0.05$) and no
282 significant differences at any post injection times were observed between them regarding the
283 effect of dietary VA level (T-test, $P > 0.05$). This is in contrast with previous reports regarding
284 *LysC* in Senegalese sole after infection with *Phdp* [34, 40].

285 Similarly to gene expression results found for *LysC*, *Prdx1* did not differ between
286 Senegalese soles fed Control and VA3 diets after the *Phdd* infection (Fig. 3; T-test, $P > 0.05$),
287 being significantly down-regulated at all sampling points after infection (ANOVA, $P < 0.05$).
288 Prdx enzymes efficiently reduce the increase in the intracellular concentration of H_2O_2 ,
289 suggesting an important function in signaling cascades by removing H_2O_2 [41] and in the
290 immunoregulation of the activity of natural killer cells (NK) [42]. Since NK cells are involved in

291 the recognition and destruction of host cells infected with pathogens, two hypotheses may be
292 proposed. The first one is in relation to the lower infection suffered by VA3 fed fish due to the
293 limited availability of iron for bacterial growth (see comments below), whereas the second
294 hypothesis is that it could represent one mechanism of evasion of the immune response by
295 *Phdd* due to the action of some proteases [43]. In contrast to *Prdx1* gene expression, we
296 reported an increased gene expression of *Lgals1* in Senegalese sole early juveniles challenged
297 with *Phdd* after 4 hpi (Fig. 3; ANOVA, $P < 0.05$), regardless of the dietary VA content. Lectins
298 are proteins that bind to specific carbohydrate moieties on cell surfaces, assisting in the rapid
299 clearance of pathogens by enhancing opsonization and phagocytosis [44]. Thus, present
300 results were in agreement with their proposed function, the removal of pathogens after the
301 onset of infection as previously found in infected fish [45].

302 Finally, we analyzed the expression of genes involved in the regulation of iron homeostasis
303 since one of the main virulence factors of *Phdd* is its high-affinity siderophore-mediated iron-
304 sequestering system [4]. The ability to acquire iron by pathogenic bacteria is essential for the
305 growth and ability to cause infections within the host. Hepcidin (*Hamp*) is a small cysteine-rich
306 protein with antimicrobial activity and an important role in iron homeostasis. Increased gene
307 expression of *Hamp* in *Phdd*-challenged Senegalese sole early juveniles from 8 hpi onwards
308 was found in this study (Fig. 3; ANOVA, $P < 0.05$). Similar results were observed in
309 Senegalese sole exposed to LPS [46]. This gene expression pattern was observed regardless
310 of the dietary VA content, with the exception of the 8 hpi sampling point, when fish fed with VA
311 supplemented diets had a significantly lower gene expression of *Hamp* than those fed the
312 Control diet (T-test, $P < 0.05$). However and in contrast to *Hamp* gene expression, *transferrin*
313 (*Transf*) gene expression was significantly up-regulated only at 24 hpi in Senegalese sole fed
314 with Control diet (ANOVA, $P < 0.05$), whereas fish fed the VA3 diet already exhibited a higher
315 gene expression value compared to the Control fish at 4 hpi. This higher level of expression
316 was maintained until 24 hpi (Fig. 3; T-test, $P < 0.05$). This increase in gene expression was in
317 accordance with the presence of a VA response element in the promoter of *transferrin* [47].

318 Since transferrin is a protein with a high affinity for iron, lowering the availability of iron in
319 vertebrate tissues, VA might inhibit bacterial growth indirectly in this way [48], and thereby
320 increase the survival of fish fed with the VA3 diet. In addition, the gene expression of *Steap4*
321 (also known as *tumor necrosis factor, alpha-induced protein 9*) was also up-regulated in fish
322 from the Control group infected with *Phdd* from 12 hpi onwards (Fig. 3; ANOVA, $P < 0.05$);
323 which was in agreement with its up-regulation in sole exposed to LPS [46]. In contrast, there
324 was a significant reduction of *Steap4* expression at each sampling point in infected fish from
325 the VA3 group (T-test, $P < 0.05$). Although *Steap4* is capable of facilitating the cellular uptake
326 of iron [49] it is induced by TNF- α as a protective anti-inflammatory factor [50] and it has been
327 reported to be down-regulated by all-trans RA (the main active VA metabolite), thereby
328 redirecting the immune balance towards immunoglobulin production and a humoral immune
329 response [51]. This was in accordance with present results regarding a reduced expression of
330 *Steap4* in soles fed with the VA3 diet from 4 hpi onwards (T-test, $P < 0.05$), concomitantly with
331 the reported higher RA levels in VA3 diet fed soles than in those fed Control diet [15].

332

333 4. Conclusions

334

335 Sole fed higher doses of dietary VA were more resistant against a bacterial challenge with
336 *Phdd*. The lower mortality rate in fish fed higher VA dietary content (203,000 total VA IU Kg⁻¹)
337 was probably due to the stimulation by RA of the immune response system, and particularly by
338 (i) avoiding cell damage and anaphylaxis by the excessive activation of the C1 complex
339 through the control of C1 inhibitor, and (ii) reducing iron availability for growth and concomitant
340 pathogenicity of *Phdd* mainly through *transferrin* and *Steap4* regulation. Furthermore, this work
341 highlights the need for integrative research on fish nutritional requirements, since the same
342 dietary VA content that hampered skeletal development [15] stimulated fish
343 immunocompetence (present study). Such opposed effects in both systems might be due to an
344 osteoimmunology cross-talk that remains to be uncovered in fish species. Finally, feeding

345 metamorphosed Senegalese sole with 203,000 total VA IU Kg⁻¹ might be an effective,
346 inexpensive and environmental-friendly method to improve Senegalese sole
347 immunocompetence while avoiding the inducement of skeletal deformities.

348

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355

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530 **Figure captions**

531

532 **Figure 1.** Fish mortality (%) during 18 days post injection of *Photobacterium damsela* subsp.
533 *damsela* strain 33539 in Senegalese sole (*Solea senegalensis*) early juveniles fed with
534 *Artemia* metanauplii containing increased levels of vitamin A (VA). Fish were fed with *Artemia*
535 enriched with 37,000, 44,666, 82,666 and 203,000 total VA IU Kg⁻¹ and infected by
536 intramuscular injection of 10 µL of *P. damsela* subsp. *damsela* suspension (6*10² CFU mL⁻¹
537 in SSW; Inf-C, Inf-VA1, Inf-VA2 and Inf-VA3, respectively); or fed with *Artemia* enriched with
538 37,000 total VA IU Kg⁻¹ and injected intramuscularly with 10 µL of SSW (Control). Different
539 letters at each day post injection denote significant differences in mortality among the different
540 dietary regimes (ANOVA; *P* < 0.05). Each experimental group was done in quadruplicate (*n* =
541 7).

542

543 **Figure 2.** Time course of relative gene expression of some molecular players of the
544 complement pathway in Senegalese sole (*Solea senegalensis*) early juveniles after injection
545 with *Photobacterium damsela* subsp. *damsela* strain 33539. Relative gene expression was
546 from fish fed with *Artemia* enriched with 37,000 (black bars; Control) or 203,000 total VA IU Kg⁻¹
547 (white bars; VA3) before (0 hours post injection; hpi) and 4, 8, 12 and 24 hpi intramuscularly
548 of 10 µL of suspension of *P. damsela* subsp. *damsela* strain 33539 (6*10² CFU mL⁻¹ SSW).
549 Different letters denote significant differences between sampling points of Control group
550 (ANOVA, *P* < 0.05). Asterisk denotes significant differences between Control and VA3 groups
551 at each sampling point (T-test, *P* < 0.05). Bars represent the relative gene expression mean
552 values of three individuals from each experimental group (biological replicates; *n* = 3). Relative
553 gene expression of each biological replicate was run in triplicate (technical replicates; *n* = 3).

554 Control fish at 0 hpi was the reference sample for relative gene expression analysis and set to
555 1. The housekeeping gene used was *ubiquitin (Ubq)*. *C1inh*, C1 esterase inhibitor; *C3*,
556 Complement component C3; *C9*, Complement component C9.

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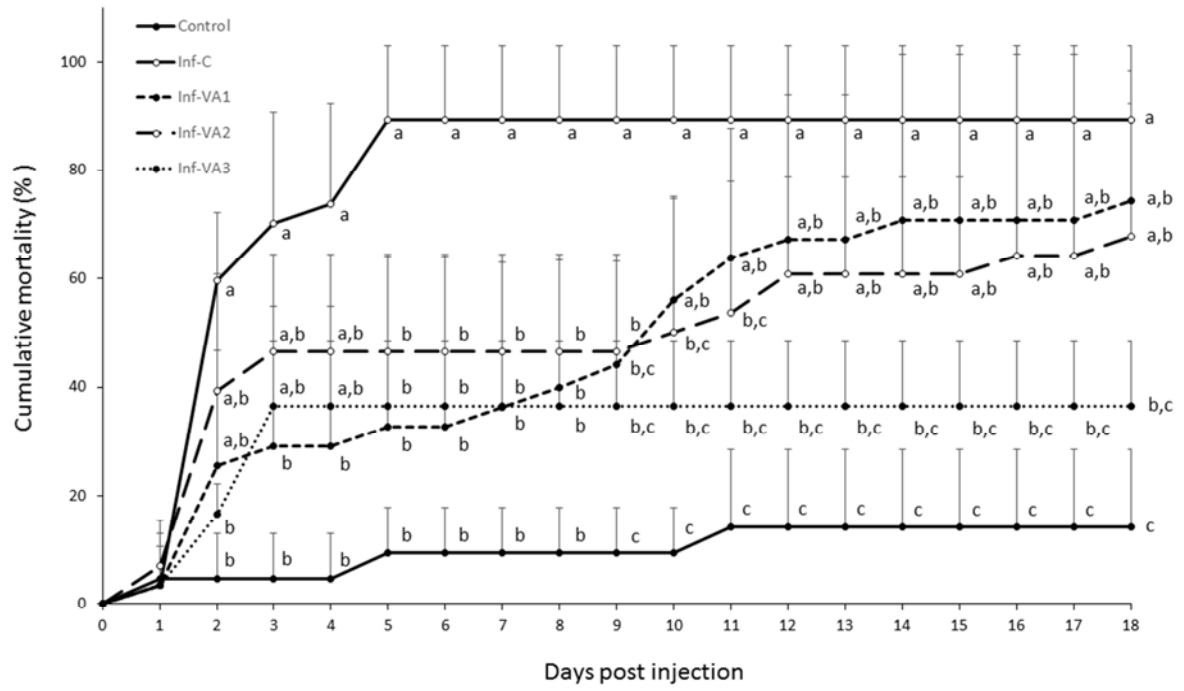
558 **Figure 3.** Time course of relative gene expression of some other molecular players involved in
559 the immune system of Senegalese sole (*Solea senegalensis*) early juveniles after injection with
560 *Photobacterium damsela* subsp. *damsela* strain 33539. Relative gene expression was from
561 fish fed with *Artemia* enriched with 37,000 (black bars; Control) or 203,000 total VA IU Kg⁻¹
562 (white bars; VA3) before (0 hours post injection; hpi) and 4, 8, 12 and 24 hpi intramuscularly of
563 10 µL of suspension of *P. damsela* subsp. *damsela* strain 33539 (6*10² CFU mL⁻¹ SSW).
564 Different letters denote significant differences between sampling points of Control group
565 (ANOVA, *P* < 0.05). Asterisk denotes significant differences between Control and VA3 groups
566 at each sampling point (T-test, *P* < 0.05). Bars represent the relative gene expression mean
567 values of three individuals from each experimental group (biological replicates; *n* = 3). Relative
568 gene expression of each biological replicate was run in triplicate (technical replicates; *n*=3).
569 Control fish at 0 hpi was the reference sample for relative gene expression analysis and set to
570 1. The housekeeping gene used was *ubiquitin (Ubq)*. *Hamp*, Hecpudin; *Steap4*,
571 Metalloreductase six-transmembrane epithelial antigen of prostate 4; *Prdx1*, Peroxiredoxin-1;
572 *LysC*, Lysozyme C; *Transf*, Transferrin; *Lgals1*, Lectin, galactoside-binding, soluble, 1 (galectin
573 1)-like 3.

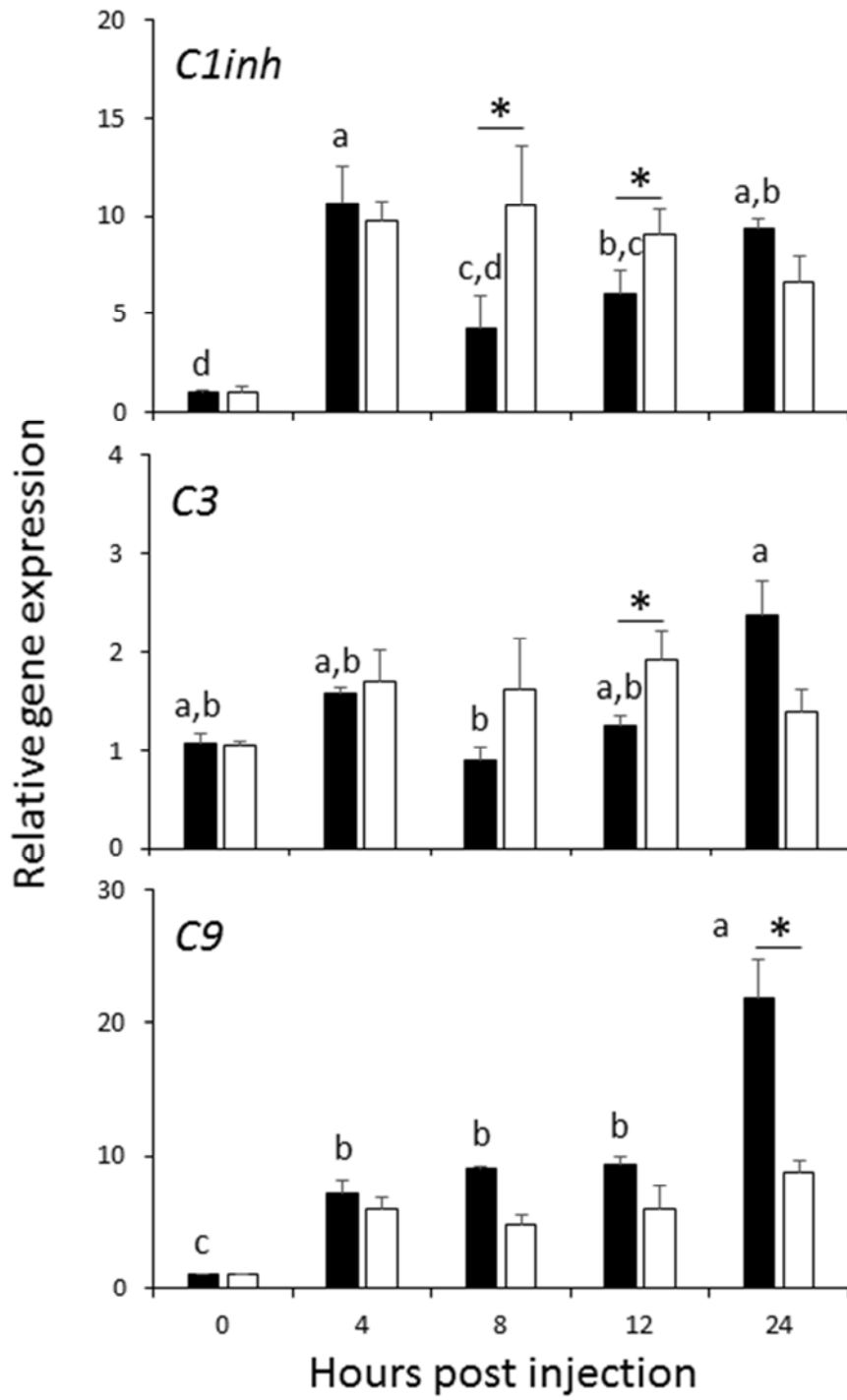
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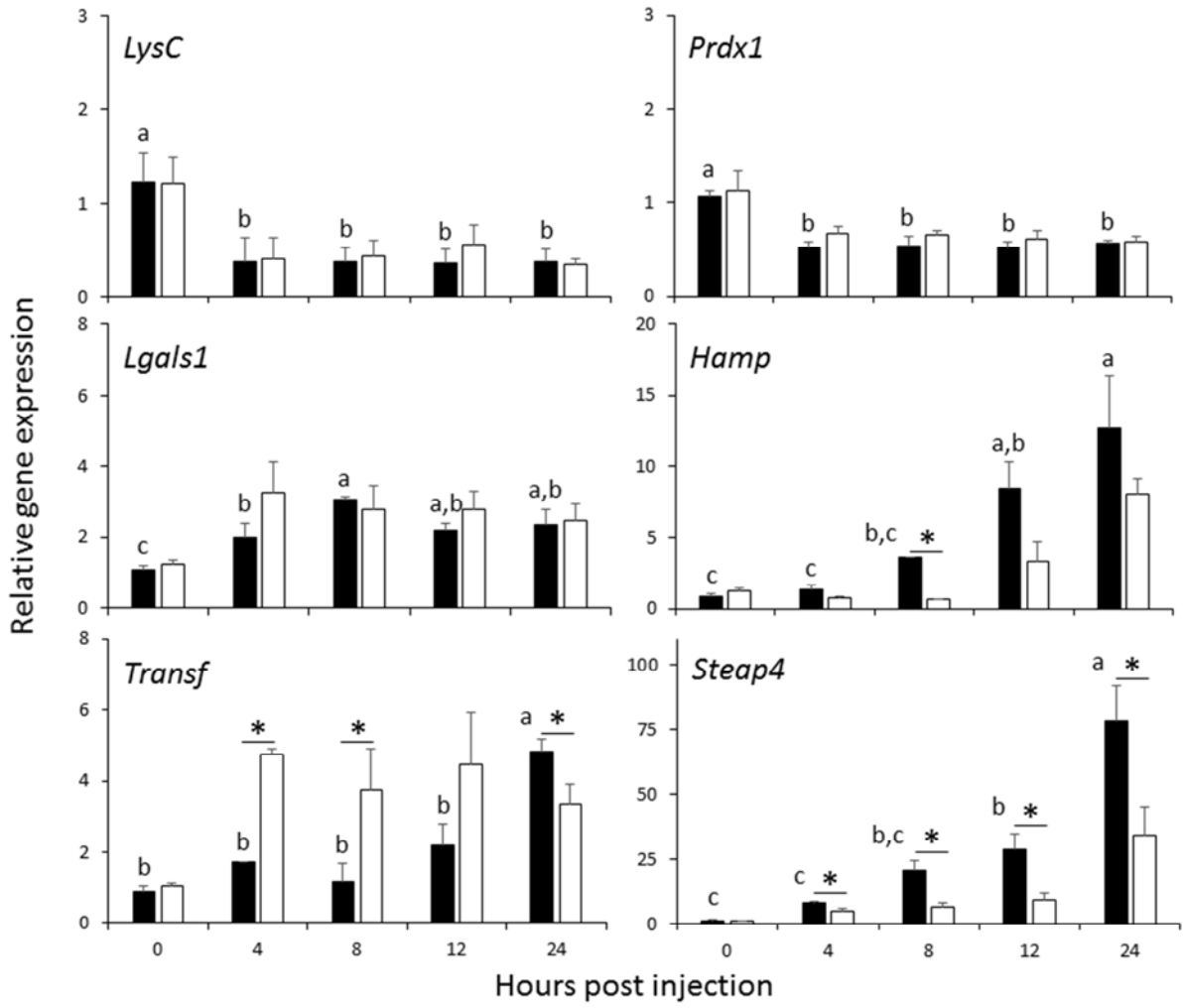
575 **Supplementary Figure S1.** Cumulative fish mortality (%) induced with intramuscular injection
576 (10 µL) of different suspensions of *P. damsela* subsp. *damsela* strain 33539 ranging from 0
577 (Control) to 6*10⁵ CFU mL⁻¹ SSW (in quadruplicates) in Senegalese sole (*Solea senegalensis*)
578 early juveniles (*n* = 10) for lethal dose 50 (LD₅₀) determination.

579

580 **Supplementary Figure S2.** Agarose gel showing PCR products amplified with 16S-like
581 ssrDNA degenerate primers from pure culture of *Photobacterium damselae* subsp. *damselae*
582 strain 33539 (B), SSW injected (C), and 4 moribund infected Senegalese sole (*Solea*
583 *senegalensis*) early juveniles (1, 2, 3 and 4). L, 1 Kb Plus DNA Ladder Invitrogen®. Note the
584 corresponding amplicon of 1,424 bp from the 16S gene of *Phdd* from the pure culture, the
585 absence of an amplicon from euthanized fish injected with SSW, and the same band in 4
586 different moribund infected juveniles. Amplicons from pure bacterial culture and infected fish
587 were sequenced, analyzed using BLAST in GenBank at NCBI and identified as *P. damselae*
588 subsp. *damselae* strain 33539.







Highlights

- Dietary vitamin A (VA) enhances immune system in *Solea senegalensis* juveniles
- Sole fed 203,000 VA IU Kg⁻¹ had higher resistance to an induced bacterial infection
- Complement system and iron homeostasis genes were differentially expressed
- Dietary VA levels impact on the fish osteoimmunological response

Supplementary Table S1. Gene name, accession numbers (GenBank or Unigene), primers and expected amplicon size used to perform the relative gene expression quantification in Senegalese sole (*Solea senegalensis*) early juveniles and confirming DNA *Photobacterium damsela* subsp. *damsela* presence in injected fish.

Gene name	Accession numbers*	Component	5' to 3' nucleotide sequences	Expected amplicon size (bp)
<i>C1inh</i>	unigene64193 unigene526786	Forward	ACCTGTGTGGCCTCTCCCCTG	107
		Reverse	ACTGGTAGCAGCAGCAGCCT	
<i>C3</i>	FJ345403	Forward	GGCTTACTAACTGGCTTCACTGTCA	110
		Reverse	CCTCTCTCTGACAGAACTGTGTCCA	
<i>C9</i>	unigene430267 unigene2945	Forward	ATCAGGAGGGTAACTGGACATGCTG	100
		Reverse	GCCCCACTCAGTCCGTCTGTGT	
<i>Lgals1</i>	unigene27503 unigene94321	Forward	TGACTCAGACAACATCGCAATGCAC	101
		Reverse	CACCCCAGCATCCCCCGGAC	
<i>Hamp</i>	FJ263548 AB455099	Forward	ACACTCGTGCTCGCCTTTGTTTGCTTC	173
		Reverse	TGACTCCAGCGTCTGTGTCTGACATGATTC	
<i>LysC</i>	DQ293993	Forward	AGCAGCTTAGGGAGAGAGACCGTC	254
		Reverse	TGTTGATCTGGAAGATGCC	
<i>Prdx1</i>	unigene65476_split_1 unigene281027	Forward	ACGGAGTGCTGAAGGAGGACGA	114
		Reverse	CCACAGAGCGACCCACAGGT	
<i>Steap4</i>	FJ263550	Forward	CTGGGACTGCTGGCACTTGGATTTG	160
		Reverse	CAGTTCTCCACGCTGACGTAATGTCGAAC	
<i>Transf</i>	FJ345407	Forward	TGCAGGGCTGGAACATTCCCATA	109
		Reverse	GCCCCGAAAAATGCACGCACG	
<i>Ubq</i>	AB291588	Forward	AGCTGGCCCAGAAATATAACTGCGACA	93
		Reverse	ACTTCTTCTTGCGGCAGTTGACAGCAC	
<i>16S-like ssrDNA degenerate</i>		Forward	AGAGTTTGATCMTGGCTCAG	1424
		Reverse	AAGGAGGTGATCCANCCRCA	

*GeneBank and/or Solea Data Base (<http://www.juntadeandalucia.es/agriculturaypesca/ifapa/aquagenet/soleaDB>) (solea_v4.1)

